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Tumor Promoting Properties of Field Cancerized Fibroblasts in Cell Culture

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TUMOR PROMOTING PROPERTIES OF FIELD CANCERIZED FIBROBLASTS IN CELL CULTURE

by

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Tumor Promoting Properties of Field Cancerized Fibroblasts in Cell Culture

By

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ABSTRACT

Our recent studies have shown that Tumor Adjacent Histologically Normal (TAHN) breast tissue demonstrates many of the characteristics of breast tumors. For example, through immunohistochemical staining with markers such as alpha smooth muscle actin and TGF-β, we have shown the accumulation of myofibroblasts in TAHN tissues 1 cm from the tumor margin (TAHN-1). Additionally, TAHN-1 epithelia stain positive for epithelial to mesenchymal transition (EMT) associated proteins, such as transforming growth factor beta and alpha smooth muscle actin. The purpose of this study was to determine if TAHN-1 fibroblasts retained myofibroblast characteristics and if they could induce EMT in primary culture. We isolated primary cells from tissue specimens taken 1cm and 5cm from a breast tumor (TAHN-1, TAHN-5). Primary fibroblasts were stained positive for myofibroblast markers, and contracted a collagen gel, demonstrating that these cells retain their myofibroblast characteristics. Primary fibroblast conditioned culture media was also able to induce EMT markers and migration normal epithelial breast cells.

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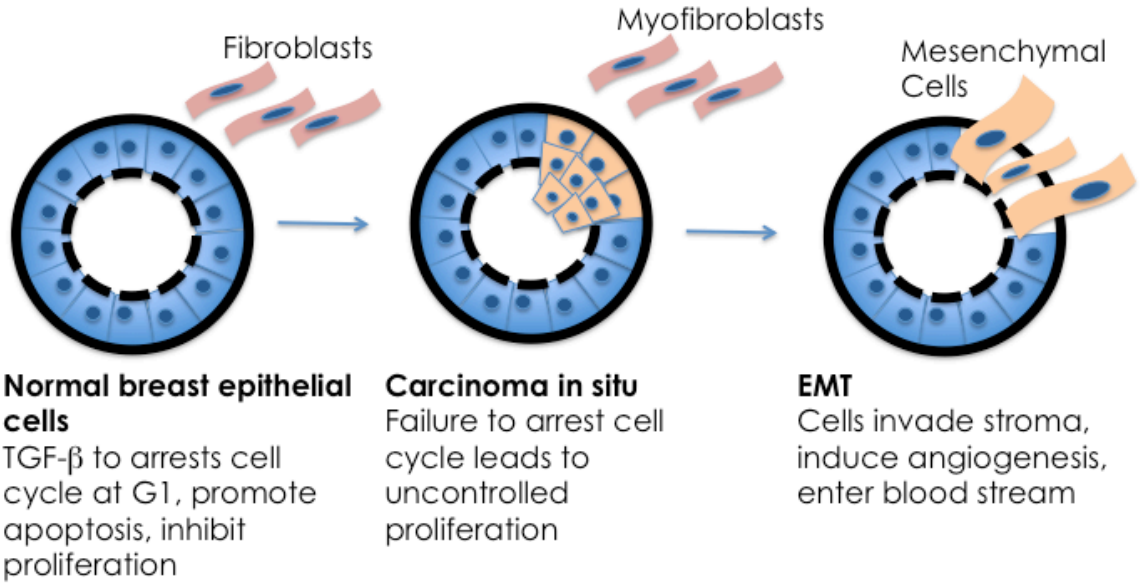
CHAPTER 1

INTRODUCTION

1.1 – Breast Cancer

Breast carcinoma originates from epithelial cells of the breast and is the most commonly diagnosed cancer amongst women.[1] Breast cancer was first thought of as a single disease, however, research has shown that breast cancer is much more complex. Breast cancer was later classified into two groups based on the expression of an estrogen receptor (ER) they were ER-positive (ER+) which are luminal and ER-negative (ER-) which are basal.[2,3] ER- can be broken down into three more subtypes: first, overexpressed human epidermal growth factor-2 (HER2); second, "normal like"; and third, triple-negative (absence of epidermal growth factor receptor (EGFR), progesterone receptor (PR), and HER2).[3] ER+ can be broken down into luminal-A and luminal-B subtypes.[3] Advancements in mammography technology and options for treatment have lead to a decrease in mortality, however there are still thousands of deaths per year due to breast cancer.[2] Research has shifted from analysis of cancer cells only to the analysis of the tumor microenvironment, which encompasses the non-cancer cells within a tumor. The breast microenvironment, or stroma, consists of inflammatory cells, vascular cells, and fibroblasts.[4] Under normal conditions, the stroma maintains proper epithelial structure, function and homeostasis.[5] As cancer progresses, epithelial cells accumulate mutations, including mutations in signaling pathways, which may result in corrupted cell signaling. The result of this mutated signaling pathway gives cancer cells the ability to suppress epithelial cell protein expression and turn on mesenchymal protein expression, ultimately

resulting in metastisis.[6] The progression from normal epithelial cells to mesenchymal cells is illustrated in Figure 1. Our studies have expanded this analysis to include the histologically normal tissue up to centimeters away from a breast tumor. Gaining a better understanding of how the tumor and stroma affect each other could help researchers and physicians achieve higher efficacy in treatments.



1 – Breast epithelial cells communicate with the stroma. Normal epithelial cells progress into carcinoma cells through several stages. Normal epithelial cells communicate with the stroma, which includes fibroblasts, to maintain proper structure, function, and homeostasis. As cancer progresses, epithelial cells accumulate mutations which can cause corruption in signaling pathways. Corrupted signaling can result in the down regulation of epithelial proteins and up regulation of mesenchymal proteins.

1.2 – Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is the process in which terminally differentiated, polar, anchored epithelial cells can transform into non-polar, motile, mesenchymal-like cells. During this process, epithelial cells lose their adherens junctions, reorganization and regulation of cytoskeletal proteins, produce extracellular matrix degrading proteins, and activation of transcription factors.[7] EMT has been shown to occur in three distinct biological settings, and has been categorized accordingly. Type 1 EMT occurs during embryonic development and organ development.[7]

Type II EMT occurs during would healing and fibrosis.[7] During this process, myofibroblasts accumulate at the site of the wound or fibrosis. These myofibroblasts release cytokines such as TGF-β and matrix remodeling proteases such as MMPs. These molecules can induce Type II EMT through the activation of the SMAD pathway. The epithelial cells become mobile to migrate into the wound bed for the process of re-epithelialization.[8] This is only a partial EMT, and the process is very regulated. Once the cell migrates into the wound, it regains its epithelial characteristics and loses its mesenchymal characteristics. It is important to note that Type I and Type II EMT do not result in neoplastic transformation.

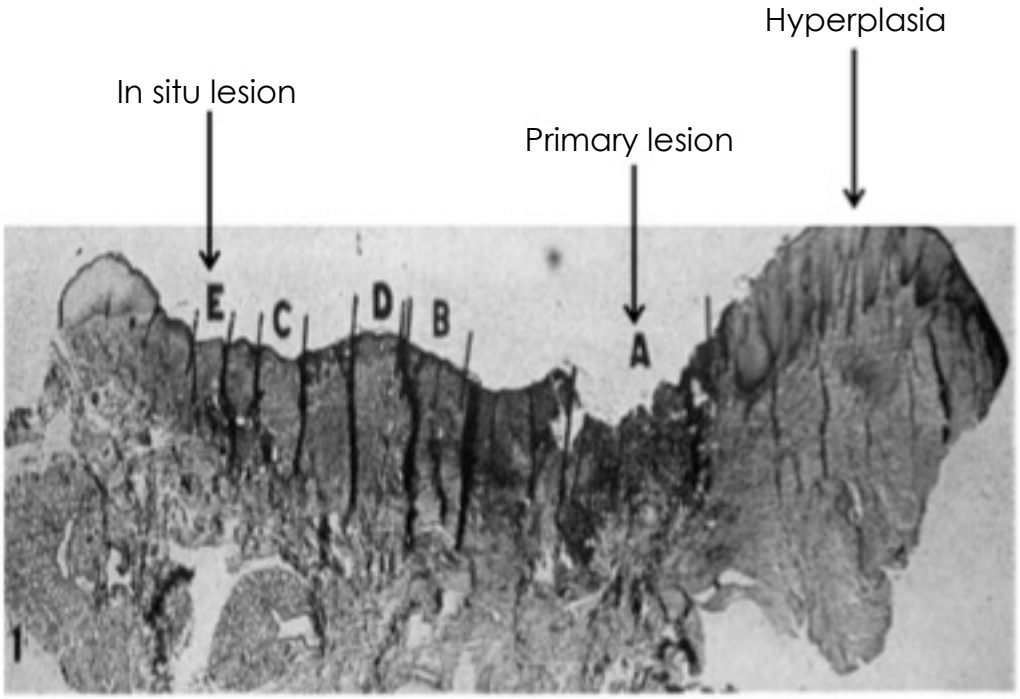
Type III EMT occurs in the context of cancer metastasis. During Type III EMT, cancer cells lose many of their adhesion molecules. They also re-arrange their cytoskeleton making them more mobile. These properties allow the cells to leave the primary tumor site, infiltrate the circulatory system and localize to a secondary tumor site where they revert to their epithelial phenotype.[7,9,10] Previous studies have shown the presence of EMT related proteins, TGF-β, SPARC (inducers of EMT), and α-SMA

(marker of EMT), in TAHN-1 and patient matched tumor human mammary tissue specimens.[11] The process of Type III EMT in tumorigenesis and metastasis occurs in a series of events. EMT is regulated by crosstalk between countless cytokines and pathways. One initiator of EMT is the overexpression of TGF-β cytokines by cells in the microenvironment. TGF-β produced by epithelial cells can also induce EMT in an autocrine fashion. During this process cells will transition from an epithelial "cobblestone" phenotype to a new mesenchymal shape.[7] This thesis will use primary human mammary fibroblasts to investigate the presence of EMT inducers in primary human mammary TAHN fibroblasts.

TGF-β acts an inhibitor of cellular proliferation in normal cells through the SMAD pathway. Additionally, high levels of TGF-β can also induce apoptosis through SMADs in non-malignant cells[10,12] . In many malignant cell types, however, SMAD pathways can accumulate mutations and lose the ability to respond to TGF-β.[12] The malignant cells then lose the ability to inhibit growth and initiate apoptosis.[6,7] This allows the cells to evade apoptosis. Additionally, TGF-β induces proliferation in these cells. Another function of TGF-β is to induce EMT. This occurs during both the process of wound healing and fibrosis (Type II EMT) and in cancer metastasis (Type III EMT)[8, 10].

1.3 - Field Cancerization

In 1953, Dr. Slaughter introduced the term "Field Cancerization" to describe the abnormalities in the tissue surrounding oral cancer lesions.[13] In this study the tissue sections revealed invasive tumors that were 1cm or smaller in size were surrounded by individual in situ carcinomas. He also observed areas of hyperplasia surrounding these

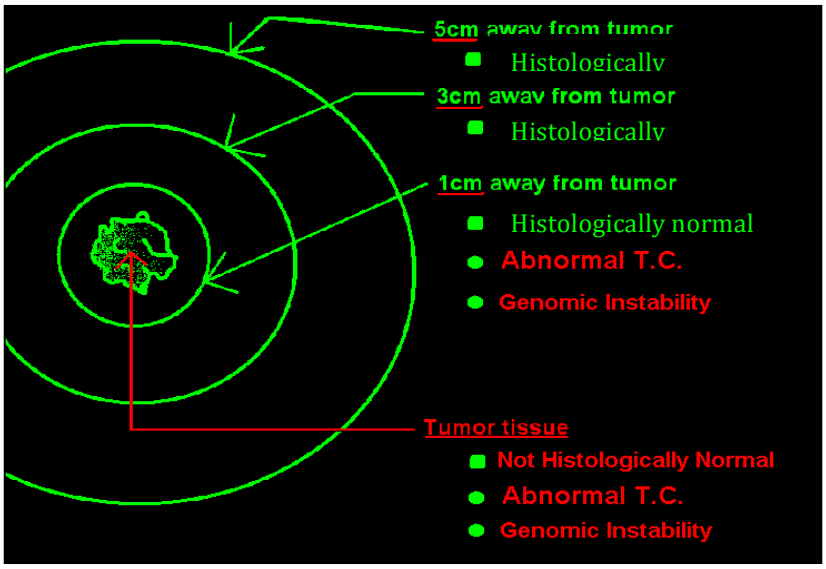


2. – Buccal mucosa photomicrograph reveals histological abnormalities surrounding a primary lesion. This section of buccal mucosa (lining of the cheeks) is from a 67 year old patient who has had 7 incidences of oral carcinoma over the span of 11 years. Point A is the absence of the primary lesion that has been excised. Points B, D, and E are in situ lesions. Point C is a small invasive carcinoma. Hyperplasia, the accumulation of epithelial cells, is at the far right of the photomicrograph. This study was done by Dr. Slaughter and is evidence that supports the field cancer theory.[13]

lesions (Figure 3). He suggested small, individual carcinomas eventually grow and combine into a large, single tumor.[13] Further analysis revealed 11.2% of oral cancer samples had two separate, primary tumors present in the same anatomical area, a statistic that is well above chance.[13] Slaughter concluded that the multifocal in situ lesions and presence of multiple primary tumors suggests a preconditioned epithelial environment responsible for the growth of a multiple tumors in the same field of tissue. He also suggested that this preconditioned epithelia, if remaining after surgery, could result in local recurrence of oral cancer.[13] It is important to note that the adjacent tissue Slaughter described was histologically *abnormal*.

Previous studies from our lab have demonstrated that *histologically normal* tissue adjacent to breast tumors possesses many of the molecular abnormalities found in patient matched tumor tissues.[11,14–16] One abnormality seen in TAHN breast tissue at 1cm from the tumor (TAHN-1) is shortened telomeres and overexpression of telomerase, not seen in patient matched TAHN tissue at 5cm (TAHN-5) from the tumor edge.[14,15] The overexpression of telomerase allows cells with short telomeres to evade apoptosis and continue proliferating. This combination leads to genetically unstable cells with the capacity for endless proliferation.[15,17] Another abnormality found in TAHN-1 breast tissue and patient matched Tumor tissue specimens is the presence of fibrosis and epithelial to mesenchymal transition (EMT) markers.[11] Previous studies have shown patient matched tumor and TAHN-1 tissue specimens accumulated markers of fibrosis, and EMT in epithelial cells, which is characteristic of many carcinomas.[11] Finally, accumulation of myofibroblasts has been observed in CAF and TAHN-1 tissue specimens.[11,18] Myofibroblasts, different from fibroblasts, are found in wound healing

events and express alpha-smooth muscle actin protein, providing myofibroblasts the ability to contract similar to smooth muscle cells.[18–20]



3. - Tumor Adjacent Histologically Normal tissue diagram. The tumor is in the center of the field with margins of histologically normal tissue margins at distances of 1cm, 3cm, and 5cm from the tumor edge. Previous studies have shown TAHN-1 tissue contains abnormal telomere content (T.C.) and genomic instability.

1.4 Implications of Field Cancerization in Clinical Practice.

Current standards for breast conserving surgeries suggest removing 1-2mm additional breast tissue surrounding the tumor when removing the tumor. This is the surgical margin.[21] However, this is a highly controversial guideline.[22,23] This results in substantial surgeon-specific variation for re-excision rates for patients (0%-70%).[24] A Meta analysis investigated margin thresholds and rates of local recurrence.[21] This study concluded that breast conserving surgeries with surgical margins of 10mm or greater had a 96% chance of better outcome, or a 0.04% chance of local recurrence.[21] However, the study also argued the larger surgical margin size may not always be necessary for samples with no cancer cells in the margin of tissue that is being removed with the tumor, called negative margins, and may leave the patient with poor cosmetic outcomes.[21] Understanding the properties of tumor adjacent tissues will help to determine if these tissue contribute to local recurrence if left behind after breast conserving surgery.

1.5 – Exosomes

Cells release a heterogeneous population of membrane bound vesicles, one type of these vesicles has been exosomes. Exosomes can be identified by their small 30-100nm size, cup shape morphology, and lipid membrane.[25,26] Once exosomes are released from the parent cell, they can be taken up by other cells through endocytosis, plasma membrane fusion, or receptor mediated fusion.[25] Exosome content is not necessarily representative of the contents of their parent cell, but is enriched in specific proteins, lipids, RNA, and DNA.[26] Recent studies have shown that pancreatic cancer cell derived exosomes contain dsDNA with mutant p53, which is a common pancreatic cancer

mutation.[27] Exosomes containing specific mRNAs were taken up by the cells of interest and successfully translated as detected by a reporter gene.[28] miRNAs have been detected in exosomes and are thought to be included for different reasons. One study suggested the inclusion of miRNAs is a way for cancer cells to remove tumor suppressor miRNA in order for cancer cells to retain a tumorigenic phenotype.[29] Another study has shown exosomes include miRNA to promote invasiveness in breast cancer cells.[30] Current exosome signaling research has focused on EMT crosstalk between cancer cells and stromal cells in the tumor microenvironment. A recent study found exosomes derived from different cancer cell lines that expressed TGF-β on the plasma membrane were able to transform normal primary fibroblasts into myofibroblasts indicated by the expression of α-SMA, a marker of EMT.[31] While most studies have focused on cancer cell exosomes, recent studies have begun to explore the effects of fibroblast derived exosomes on epithelial cells to examine the crosstalk between epithelial cells and the stroma. This thesis will focus on the influence THAN fibroblasts have on normal epithelial cells measured by the accumulation of EMT markers, TGF-β and SPARC, which EMT may be mediated through.

1.6 Rationale

Based on previous studies using TAHN tissue specimens, we expect to see EMT markers retained in primary cell culture, TAHN-1 fibroblasts to have wound-healing abilities similar to CAF, and TAHN-1 fibroblasts induce EMT in normal breast epithelia. The central hypothesis of this thesis is TAHN-1 fibroblasts retain EMT markers in primary cell culture which may induce EMT in normal breast epithelia.

CHAPTER 2

METHODOLOGY

2.1 Tissue processing

Fresh human mammary tissue specimens derived from the tumor and margins of 1cm, 3cm and 5cm from tumor were acquired from University of New Mexico Human Tissue Repository. Specimens were immediately minced and enzymatically digested in fibroblast culture media supplemented with 10% collagenase-A (Worthington, Lakewood, NJ) for 18 – 20 hours at 37C with agitation. At completion of digestion, specimens were centrifuged at 1500 RPM for 10 minutes to pellet the resulting small tissue fragments needed for primary cell culture.

2.2 Primary Cell culture

Small tissue fragments produced by digestion were pelleted and digestion media was transferred to fresh tube and centrifuged to pellet single cells. The tissue fragments and single cells were plated separately in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Sigma-Aldrich) and 1% Pen-Strep antibiotics (Life Technologies, Grand Island, NY). Cells were maintained at 37C with 5% CO2 in a humidified environment.

2.3 Cell Lineage Culture

Non-malignant MCF-10A breast epithelial cells (ATCC, Manassas, VA) were cultured in F-12 DMEM medium (Life Technologies) supplemented with 50 nM

hydrocortisone (Sigma-Aldrich), 20 ng/ml EGF (Sigma-Aldrich), 0.01 mg/ml human insulin (Sigma-Aldrich), 5% fetal bovine serum (FBS) (Sigma-Aldrich), and 1% Pen-Strep (Life Technologies). Cells were montained at 37C with 5% CO2 in a humidified environment.

2.4 Immunocytochemistry

Cells were seeded on sterile glass coverslips overnight. The next day, culture media was aspirated and cells were washed twice with PBS. Cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes at room temperature. Cells were washed twice and then permeabilized with 0.2% triton (Sigma-Aldrich) in PBS for 5 min at room temperature. Cells were washed twice with PBS and Blocked using 5% BSA (Sigma-Aldrich) in PBS for 1 hour at room temperature. Blocking media was aspirated and primary antibodies were applied for I hour at room temperature. Rabbit alpha-smooth muscle actin 1:200, mouse transforming growth factor – beta 1:1000, and rabbit SPARC 1:500 (Abcam, Cambridge, MA) were diluted in blocking media. Cells were washed twice with PBS and secondary antibodies were applied for 30 minutes at room temperature. Secondary antibodies were chicken anti mouse AlexaFluor 488 and donkey anti rabbit AlexaFluor 633 (Life Technologies). Cover slips were mounted to glass slides using ProLong Gold mounting media with DAPI (Life Technologies). The slides were imaged by a Zeiss LSM510 and Zen 2009 at 50x.

2.5 Collagen Contraction Assay

Primary human fibroblasts at 2.0 x 10^4 cells/well were suspended in a 1.3 mg/ml collagen-I solution (BD Biosciences, San Jose, CA) according to manufacturer's protocol. Immediately incubate culture plate at 37C with 5% CO2 after cells were seeded in the collagen suspension. Images of the wells were acquired using a dissecting microscope in order to image as much of the well as possible. The area of the collagen being contracted was measured and quantified using imageJ.

2.6 Migration Assay

MCF-10A cells were seeded at 90% confluence overnight. Confluent cells were scratched to remove an area of cells and culture media was replaced with conditioned media. Conditioned culture media is collected from primary fibroblasts and contains fibroblast derived exosomes. Conditioned media is centrifuged at 10,700 RPM at 4C for 45 minutes to pellet exosomes resulting in exosome depleted conditioned media. Cells were placed in the Incucyte Zoom from Essen Bioscience (Ann Arbor, MI). Images were taken every hour for 48 hours.

2.7 Conditioned Media Induced EMT

MCF-10A cells were seeded at 30% confluence overnight on sterile glass coverslips. Cell culture media was replaced with primary fibroblast conditioned culture media for 24 hours. Cells were fixed, permeabilized, and stained using Immunocytochemistry described above. Statistics were conducted using a two-sided student's t-test in R.

2.8 Exosome Isolation

Exosome enriched culture media from primary human mammary fibroblasts was collected and stored at 4C. Differential ultracentrifugation was performed to purify the exosomes from cell debris. Media was centrifuged at 300g for 10 min, the pellet was discarded and the supernatant was transferred to a fresh tube. Supernatant was centrifuged at 2,000g for 10 min, the pellet was discarded and the supernatant was transferred to a fresh tube. Supernatant was centrifuged at 10,000g for 10 min, the pellet was discarded and the supernatant was transferred to a fresh tube. Supernatant was centrifuged at 100,000g for 70min; the supernatant was carefully discarded, leaving the exosome "pellet". The exosome pellet was washed with PBS. The exosomes were centrifuged at 100,000g for 70min. PBS was carefully removed to not disturb the exosome pellet. The exosome pellet was suspended in 300ul PBS and stored at 4C.[32]

2.9 RNA

Pelleted exosomes were used with the Total Exosome RNA and Protein Isolation kit from Life Technologies, following the manufacturer's protocol. Briefly, exosomes that were pelleted using ultracentrifugation, previously described, were incubated on ice for 5 minutes with 1 aliquot of 2X Denaturing Solution. After incubation, the volume of Acid-Phenol:Chloroform added was the same as the total sample plus previously added solutions. Samples were vortexed for 1 min followed by centrifugation at maximum speed for 5 min at room temperature. The upper, aqueous phase is carefully removed and transferred to a new tube. 100% EtOH is added to the collected aqueous phase and mixed

thoroughly. The sample was then transferred to a spin column and collection tube and centrifuged at 10,000g for 15 seconds or until the sample passed through the column. Discard the flow-through. miRNA Wash Solution was added to the filter and centrifuged as in previous step. Discard the flow-through. Preheated Elution Solution is applied to the filter, which was placed in a new collection tube. Recover the RNA by centrifugation for 30 seconds. Do not discard the flow-through. RNA samples were quantified using the nanodrop (NanoDrop, Wilmington, DE). miRNA samples were sent to Exiqon (Woburn, MA) for miRNA analyses.

2.10 Transmission Electron Microscopy

Exosome suspensions were incubated for one hour at room temperature on carbon film-coated copper grids; the grids were glow discharged prior to sample mounting. After sample adhesion to the support films, the grids were washed twice on 50 ul water droplets, then stained for two minutes with 2% uranyl acetate stain. Stain was removed by wicking, and the grids were air dried. The grids were imaged in a Hitachi H7500 TEM using an AMT XR60 camera.

2.11 Statistical Analysis

Because the collagen contraction assay involved many variables and patient variability, help from a statistician was required. A linear mixed model was used to predict the contracted area of collagen gel by accounting for both fixed and random effects. Fixed effects were variables that assumed only specific values and did not change over time such as day and source. Random effects were those that varied due to inherent

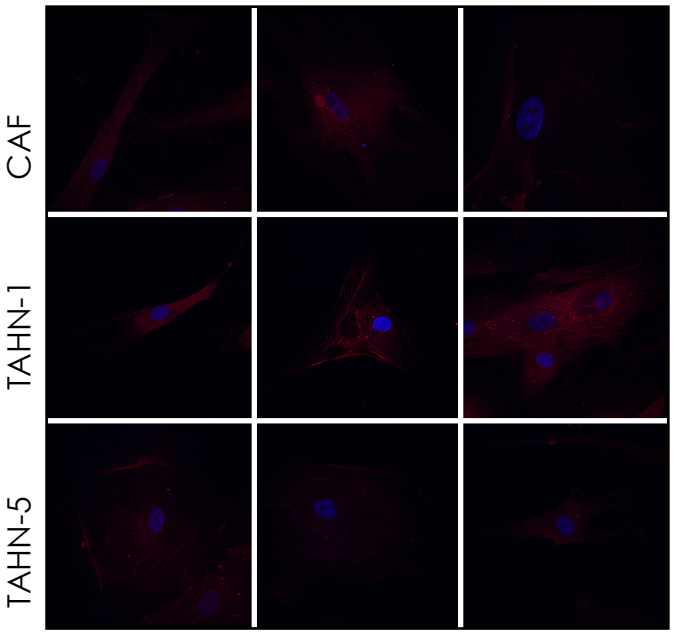
randomness in the sample such as patient and the well in which the sample was plated (the same well number varied by plate). The model assessed changes within fixed effects such as sample sources (CAF, TAHN-1, and TAHN-5), and time points of the measurements across the 6 patients (the random effects). Least squares means were used to estimate the mean area of the samples. The differences of the least squares means were calculated to determine whether statistically significant changes in area contraction occurred between the measurement of a source at a fixed time point against a different measurement of a source at a fixed time point. The p-values for these differences were compared to a Bonferroni-adjusted type I error rate of α=0.0042. The Bonferroni correction was determined by dividing the original type I error rate (α = 0.05) by the number of sources and time points being tested (12).

CHAPTER 3

RESULTS

3.1 TAHN Fibroblasts Express α-SMA

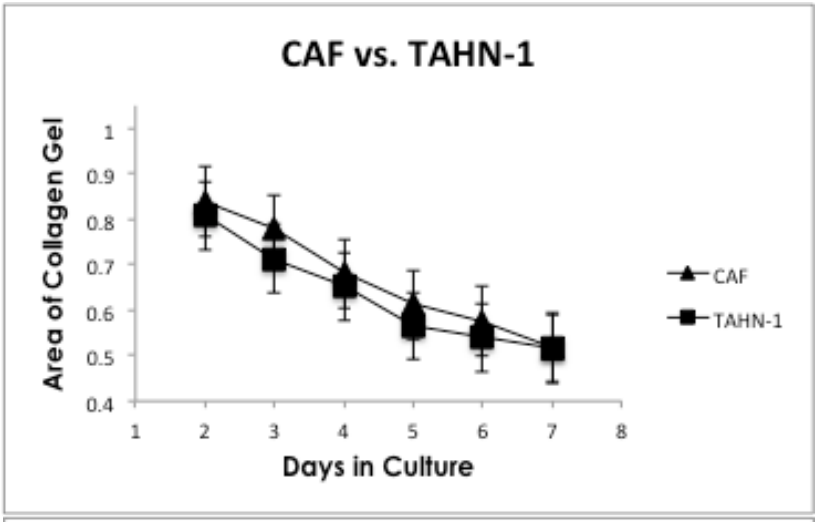
Fibroblasts derived from a tumor are cancer Associated Fibroblasts (CAFs) and share many properties with myofibroblasts such as α-SMA expression and the ability to contract. Previous studies have shown human mammary tissue specimens from cancer and TAHN-1 margins exhibit high numbers of myofibroblasts, which is not seen in TAHN-5.[11] We predicted primary cell culture of CAF and TAHN-1 fibroblasts would retain myofibroblast phenotype, which would not be seen in TAHN-5 as seen in previous studies that used tissue specimens. To test this, primary fibroblasts were grown on glass cover slips and stained with α-SMA antibody, a marker of myofibroblasts. Representative images are shown in Figure 2. CAF and TAHN-1 panels demonstrate the α-SMA stress fibers characteristic of myofibroblasts, which is not present in TAHN-5 panels. We can tell that these are myofibroblasts and not another smooth muscle cell due to the lack of α-SMA accumulation in the TAHN-5 images.

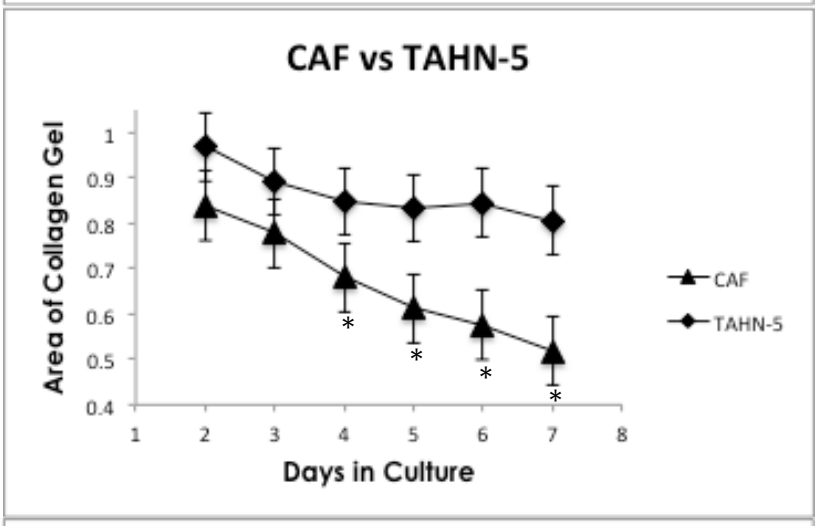


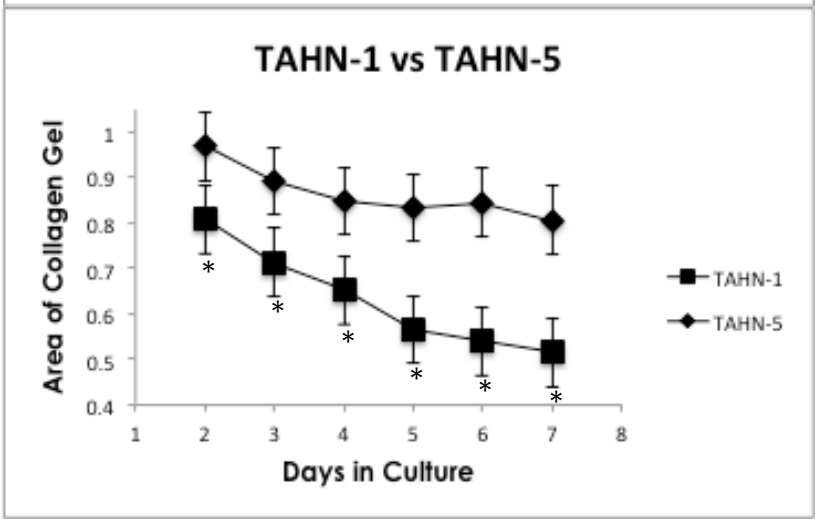
4. - Immunocytochemistry staining of primary fibroblasts demonstrate the presence of α-SMA, a myofibroblast marker (red). Nuclei are identified using DAPI (blue). n = 1.

3.2 Primary Fibroblasts Exhibit Collagen Contraction Characteristics.

Myofibroblasts play a critical role in the process of wound healing. One of the main functions of myofibroblasts in wound healing is to contract the edges of the wound together. After identifying α-SMA in the primary TAHN fibroblasts, we tested their contraction ability by culturing the cells in a collagen suspension. Images of the collagen gel were taken daily for one week to track changes in the area of the gel. The area of collagen was measured and quantified using ImageJ. Data is plotted as the ratio of the original area to the area at the measured time point. Statistical analysis reveals that patient matched CAF and TAHN-1 fibroblasts contract more than TAHN-5 and TAHN-1 fibroblasts contract more than CAF (Fig. 3). Taken together the result of this analysis is the fibroblasts in CAF and TAHN-1 cultures are myofibroblasts seen by the ability to contract.[18,19] This supports the theory that the cancer microenvironment behaves similar to an over healing wound.[33] Linear mixed model statistics were used to accurately measure the effects of time and contracted area along with the randomness introduced by the patient to patient variability for 6 patient sets (n=6). Least square means statistics was used to transform the calculations from the linear mixed model into an interpretable equation that can be used to predict the points that can further be interpreted into graphical data.



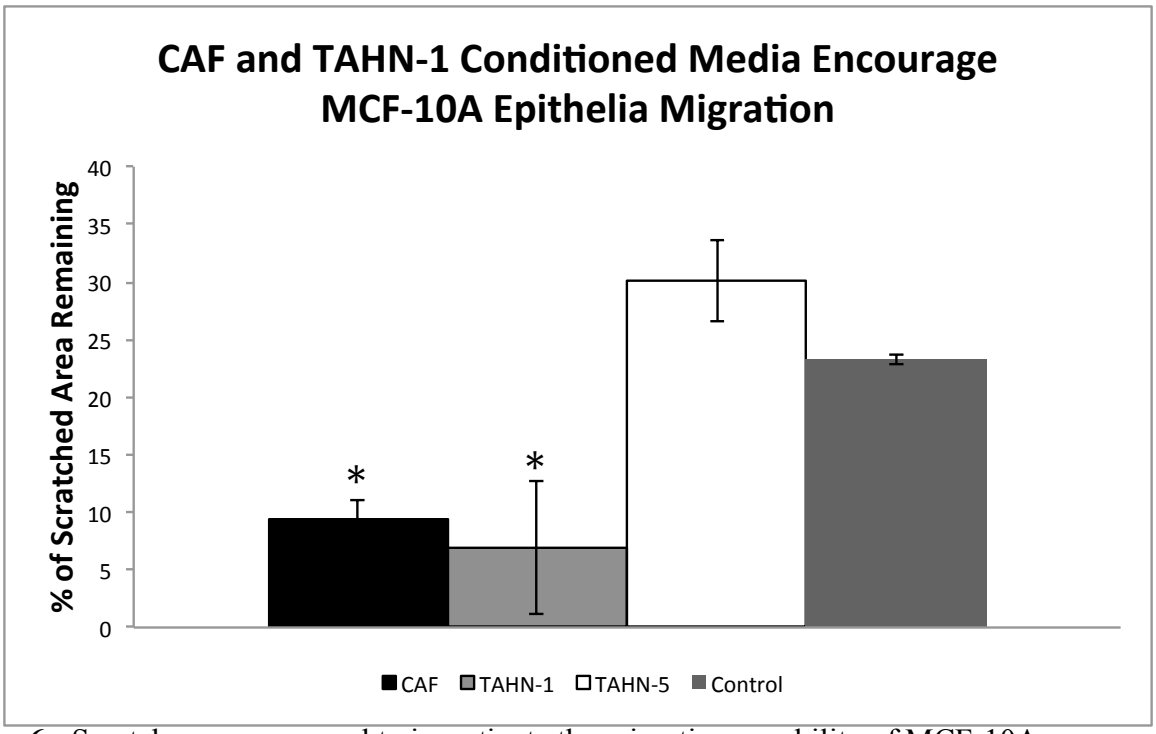




5 - Collagen contraction assay to measure the ability of CAF and TAHN fibroblasts to contract a collagen gel. Data is plotted as the ratio of the original area of the collagen gel over the area at the measured time point. Linear mixed model and least square means statistics were used to determine significance. Error bars represent confidence intervals. (*)Indicates the measurement is statistically different from the paired TAHN-5 sample $p<0.005$

3.3 CAF and TAHN-1 Exosome Enriched Conditioned Media Increases MCF-10A Cell Migration.

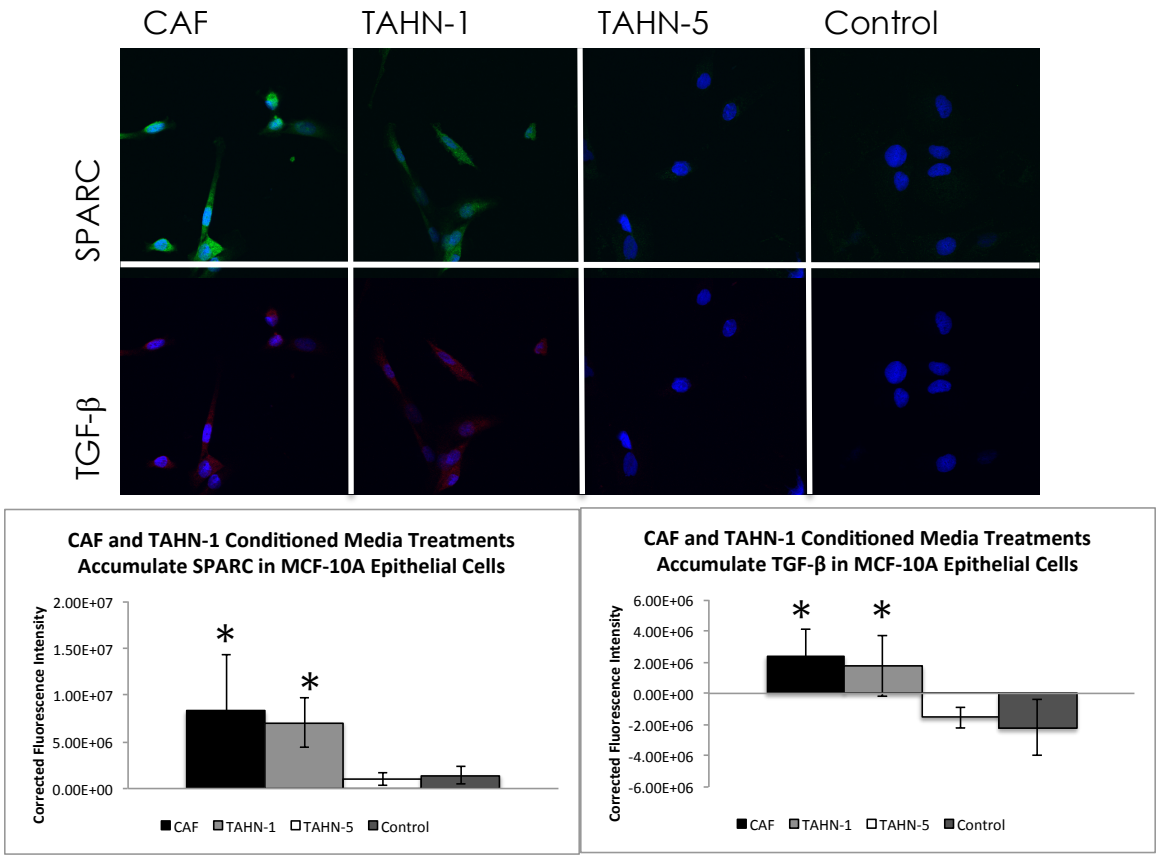
A hallmark of cancer is the ability of a cell to migrate from its primary location and metastasize in a new location.[9,34] We investigated the effects of conditioned CAF and TAHN culture media, containing exosomes, as a treatment for MCF-10A cells. Exosome enriched conditioned medium was collected from primary CAF and TAHN fibroblasts at 48 hours after cell passaging. MCF-10A cells were seeded at 90% confluence over night. A scratch was made through the middle of the monolayer and MCF-10A cell culture media was replaced with conditioned cell culture media or fresh fibroblast culture media for 48 hours. The plate was placed in the IncuCyte live cell imager and images were taken every hour for 48 hours. ImageJ was used to measure the area of the scratches. A two-sided t-test was used to determine the statistical significance between CAF and TAHN-1 areas of migration compared to TAHN-5. The statistics reveal CAF and TAHN-1 both have more cells that migrated into the scratch compared to TAHN-5 observed by the decreased area of the scratch (Figure. 4).



6 - Scratch assay was used to investigate the migration capability of MCF-10A epithelial cells when treated with conditioned culture media. Data is plotted as the area of the scratch after 48 hours of treatment. Bars are the standard deviation. (*) Indicates the measurement is statistically different from the paired TAHN-5 sample $p<0.005$, $n = 3$.

3.4 EMT Markers are Seen in MCF-10A Epithelial Cells That Have Been Treated with CAF and TAHN-1 Conditioned Media.

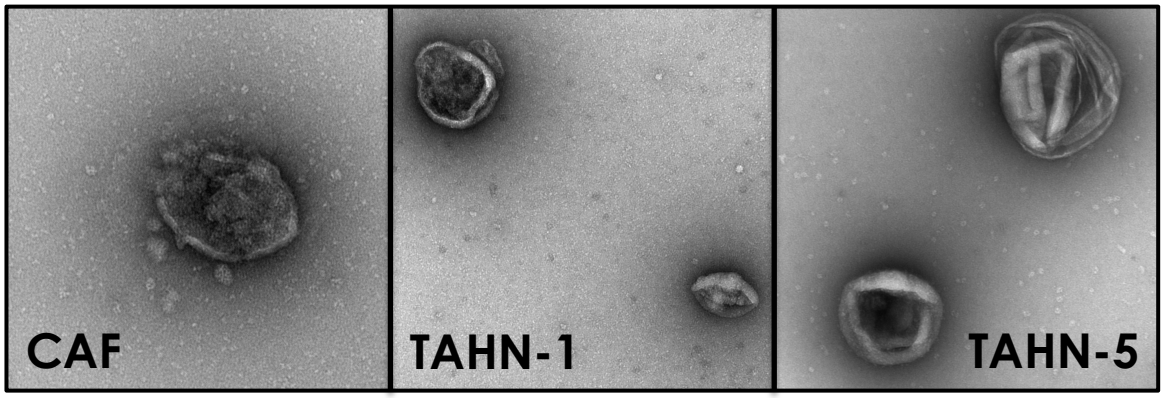
After seeing CAF and TAHN-1 myofibroblasts stain positive for α-SMA, demonstrate wound-healing capabilities, and encourage migration, we set out to ask if EMT could be induced by CAF and TAHN fibroblasts in MCF-10A epithelia. Conditioned cell culture media was collected from primary CAF and TAHN fibroblasts at 48 hours after cell passaging. MCF-10A cells were seeded onto sterile glass coverslips over night. MCF-10A cell culture media was replaced with conditioned cell culture media or fresh fibroblast culture media for 24 hours. We have previously observed SPARC (a secreted protein that has been found to induce EMT)[35,36] and TGF-β in epithelial cells undergoing EMT in TAHN-1 tissues. Immunocytochemistry using antibodies against SPARC and TGF-β, was used to determine if CAF and TAHN conditioned media could induce these proteins in MCF-10A cells. The corrected fluorescence intensity was used to quantify the amount of fluorescence without any background interference for the entire field using ImageJ. A two-sided t-test was used to determine the statistical significance between CAF and TAHN-1 compared to TAHN-5. The statistics reveal CAF and TAHN-1 conditioned media caused significant elevation of both SPARC and TGF-β in MCF-10A cells, which is not seen in TAHN-5 or the control (fresh culture medium) treatments.



7 - Immunocytochemistry was used to detect the presence of EMT proteins, SPARC (green) and TGF-β (red) in conditioned media treated MCF-10A cells. Nuclei are identified with DAPI. (*) Indicates the measurement is statistically different from the paired TAHN-5 sample $p<0.005$, $n=1$.

3.5 Fibroblast Conditioned Cell Culture Media Contains Exosomes

Earlier experiments demonstrate the ability of TAHN-1 fibroblasts to initiate EMT similar to CAF but not seen in TAHN-1. One possible method of signaling these alterations may be due to exosomal communication. Here we test for the presence of exosomes in fibroblast conditioned culture media. Fibroblasts were cultured in media supplemented with pre-manufactured exosome depleted FBS. Conditioned media was collected at 48 hours after passaging and differentially ultracentrifuged as previously described. Data were generated in the UNM Electron Microscopy Shared Facility supported by the University of New Mexico Health Sciences Center and the University of New Mexico Cancer Center. (Fig. 6).



8. - Transmission electron microscopy reveals exosomes are released from primary fibroblasts into culture media.

CHAPTER 4

DISCUSSION

There is sufficient evidence showing tumor adjacent tissue can have molecular alterations in the absence of histological abnormalities. Previous studies report shortened telomeres, genetic instability, and uncontrolled proliferation.[15,14] There is also evidence of the presence of myofibroblasts in histologically normal tissues 1cm from the tumor. Markers of EMT have also been seen in TAHN tissue specimens at a margin of 1cm away from the tumor edge.[11] Previous studies have shown that myofibroblasts can induce an EMT phenotype in epithelial cells. Based on these observations, we hypothesized that myofibroblasts observed TAHN-1 specimens would maintain their myofibroblast phenotype in primary culture, along with their ability to induce EMT in epithelial populations. We have also begun to investigate a mechanism through which this occurs.

The first conclusion of these studies is that TAHN-1 fibroblasts maintain myofibroblast markers, as well as myofibroblast functional characteristics when removed from the context of the tissue and grown in primary culture. Myofibroblasts are mesenchymal cells, which have contraction and migration capabilities. Immunocytochemistry with α-SMA indicates TAHN-1 but not TAHN-5 cells retain this myofibroblast marker. Myofibroblasts are associated with solid tumor cancers and enhance tumor progression by secreting ECM altering proteins.[7,31] A contraction assay demonstrates the ability of TAHN-1 cells, but not TAHN-5, to contract the edges of the collagen culture. An interesting finding in this test was the ability of TAHN-1 to contract to a higher degree than CAF. It is also important to note that TAHN-1 contracted a larger area than THAN-5 even when 1-2mm surgical margins are considered to be normal.

Although we show that myofibroblast protein alpha-smooth muscle actin accumulates in TAHN-1 and CAF cells we need to test for the presence of smooth muscle proteins to conclude they are true myofibroblasts.[20,37,38]

The second conclusion of this study is that TAHN-1 fibroblasts can induce EMT in non-malignant MCF-10A epithelia through secreted factors. Previous studies show EMT markers in TAHN tissue tested by immunohistochemistry.[11] Immunohistochemistry is a tool to visualize the presence of EMT markers in tissue, it cannot be used to determine how these properties were induced.[11] Two of the EMT-related proteins we observed in epithelial cells in TAHN-1 tissues were SPARC and TGF-β. To test if these markers could be induced by TAHN-1 fibroblasts, conditioned culture media from patient matched CAF, TAHN-1, and TAHN-5 fibroblasts were used as a treatment to test if EMT properties were being communicated from stromal cells to epithelial cells.

Immunocytochemistry using antibodies against SPARC and TGF-β show CAF and TAHN-1 treated MCF-10A stain positive for these EMT markers. These markers cannot be seen in TAHN-5 treated MCF-10A. TGF-β is used in a myriad of signaling pathways one of which is the SMAD pathway, which can initiate α-SMA expression as a downstream effect. [12,31] Similar to TGF-β, SPARC is involved in many signaling pathways. Previous studies show over expression of SPARC can cause EMT in melanoma and is an indicator of poor prognosis of breast cancer.[35]

To test if the functional EMT characteristics can be induced by TAHN-1 conditioned media. A migration assay was performed on MCF10a cells treated with fibroblast conditioned media. We see CAF and TAHN-1 treated MCF-10A cells are able to migrate and close the wound scratch more that TAHN-5 and control. This is in

accordance with the SPARC and TGF-β staining. TGF-β and SPARC are proteins that are understood to induce a migratory characteristic.[12,35]

Taken together, this study validates our hypothesis that TAHN-1 myofibroblast retain the myofibroblast phenotype and can induce EMT in cell culture. First we demonstrated that TAHN-1 fibroblasts, when removed from the context of the tissue, retain their myofibroblast markers and demonstrate functional myofibroblast characteristics such as contraction. Additionally, we demonstrated that TAHN-1 fibroblasts could communicate to normal epithelial to undergo an EMT transformation. This was shown both though staining with EMT markers, and through functional migrations assays. These characteristics suggest TAHN-1 specimens may be more closely related to CAF than TAHN-5 fibroblasts. This study provides new insight into understanding tumor adjacent field tissue and what may be causing local recurrence in breast cancer. This study is similar to the study done decades ago by Dr. Slaughter by showing abnormalities in tumor adjacent fields of tissue. The main difference would be our molecular abnormalities are found in histologically normal tissue, whereas, Dr. Slaughter studied histologically abnormal specimens.

There is sufficient evidence supporting EMT initiation in the tumor microenvironment via exosome communication.[29,29,31,39] Transmission electron microscopy reveals exosomes are present in primary cell culture media, but does not reveal what cargo is being transported within the exosomes. Studies are currently being done to investigate the contents of the CAF and TAHN fibroblast derived exosomes. We are testing the affects of exosome depleted conditioned media may have on migration and EMT induction on MCF-10A breast epithelial cells.

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